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LOUISIANA STATE UNIV MEDICAL CENTER NEW ORLEANS
THE ROLE OF LIMITED PROTEOLYSIS OF THYROTROPIN-RELEASING HORMON--ETC(U)
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pressure liquid chromatography were similar. An analysis of the regional distribution of cyclo (His-Pro) and TRH in rat and monkey brains exhibited no apparent precursor-product relationship. Studies on the neuroanatomic sites for the thermoregulatory effects of cyclo (His-Pro) suggested that the neural loci responsible for cyclo (His-Pro)-induced hypothermia resides within POA/AHA. The endogenous levels of brain cyclo (His-Pro) were elevated when rats were made either hypothyroid by surgical thyroidectomy or forced to drink alcohol for six weeks. These studies demonstrate that cyclo (His-Pro) is present throughout the central nervous system in physiologically relevant concentrations which can be modified by appropriate physiological and pharmacological manipulations. These data in conjunction with earlier reports of multiple biological activities of exogenous cyclo (His-Pro), suggest that endogenous cyclo (His-Pro) is a biologically active peptide and it may play a neurotransmitter or neuromodulator role in the central nervous system.

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FINAL REPORT

THE ROLE OF LIMITED PROTEOLYSIS OF THYROTROPIN-RELEASING HORMONE
IN THERMOREGULATION

Chandan Prasad, Ph.D.
Associate Professor of Medicine
and Biochemistry
Louisiana State University Medical Center
New Orleans, Louisiana 70112
CONTRACT # N00014-80-C-0416



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Thyrotropin-releasing hormone (TRH) is a hypothalamic hormone known to possess a number of endocrine and central nervous system (CNS) related functions. TRH attenuates the hypothermic effects of a number of CNS active compounds, and also produces hyperthermia when administered exogenously. The limited proteolysis of TRH by pyroglutamate aminopeptidase from CNS results into formation of a new cyclic dipeptide, cyclo (His-Pro) Histidyl-proline diketopiperazine. This new cyclic dipeptide has profound effects on thermoregulatory physiology; it not only produces hypothermia, but also antagonizes the hyperthermic effects of TRH.

In order to evaluate the role and the mechanism of thermoregulatory effects of these two peptides, several lines of investigations were pursued during the tenure of this contract. The results of these studies are summarized below:

I. Mapping Neuroanatomic Sites for the Hypothermic Effects of Cyclo (His-Pro)

Intraventricular injection of 1 μ mole cyclo (His-Pro) (1 μ l/rat) produced a hypothermia of 1.6⁰ C which lasted for 20-40 minutes. In order to determine the site of hypothermic action of cyclo (His-Pro), 1 μ mole cyclo (His-Pro) in 0.5 μ l saline was infused over a one minute period into various hypothalamic and extra-hypothalamic loci of unanesthetized, unrestrained Sprague-Dawley rats bearing chronic cannulae. Rectal temperatures were recorded with a tele-thermometer (Yellow Spring, Ohio). The thermometer probe was inserted 6 cm into the rectum and held in place for 45 seconds before temperature was recorded. Temperatures were recorded every 10 minutes before injection until a stable rectal temperature was achieved and then every 10 minutes after the injection. Each animal served as its own control by being retested with either vehicle or test substance after a 4 day recovery period.

Figure 1 is a composite of brain sections showing the injection sites tested. Hypothermic response to infusion of cyclo (His-Pro) was always positive in the preoptic area/anterior hypothalamic area (POA/AHA). On the other hand, core temperature did not decrease significantly below control level after injection into the posterior hypothalamic area (PHA) medial hypothalamic area (MHA) or

hippocampus. The average change in rectal temperature following injection in the POA/AHA was $-1.14 \pm 0.13^{\circ}\text{C}$ vs. a change of $+0.14 \pm 0.09^{\circ}\text{C}$ ($p < 0.01$) for saline treated controls. These results show that the neural loci responsible for cyclo (His-Pro)-induced hypothermia reside within POA/AHA. Furthermore, intraventricularly injected (^3H -Pro)-cyclo (His-Pro) was found to concentrate in POA/AHA (% of total radioactivity, POA/AHA = $62.4 \pm 4.7\%$, MHA = $22.5 \pm 3.3\%$, PHA = $15.0 \pm 1.4\%$ and median eminence = $1.9 \pm 0.5\%$, $N = 5$).

II. Development of Cyclo (His-Pro) RIA and its Measurement in Rat and Monkey Brain

A. Specific RIA of Cyclo (His-Pro)

We have recently developed a specific RIA for cyclo (His-Pro). Rabbit anti-cyclo (His-Pro) antisera were raised by a procedure similar to that described for the production of TRH antibody except that a cyclo (His-Pro)-bovine serum albumin conjugate was injected subcutaneously. The data presented in Table 1 shows the high specificity of our cyclo (His-Pro) antibody. The antiserum failed to react with two hypothalamic hormones (TRH and luteinizing-hormone releasing hormone), two TRH metabolites (pGlu-His-Pro and pGlu-His), constituent amino acids (L-histidine and L-proline), and two analogues of cyclo (His-Pro), cyclo (Pro-Gly) and cyclo (Ala-Gly). Cyclo (His-Pro) cross-reacted only minimally (0.781%). A typical standard curve for cyclo (His-Pro) RIA is shown in Fig. 2. Significant tracer displacement was affected by 10 pg cyclo (His-Pro) per tube, and 50% displacement was produced by 320 pg. The useful range of the standard curve extended to about 2.56 ng. The intraassay coefficient of variation derived from three replicate samples containing 10 to 1280 pg cyclo (His-Pro) per tube was $4.4 \pm 0.6\%$. The interassay co-efficient of variation derived from six independent assays of aliquots of the same sample was $10.0 \pm 0.6\%$. The addition of rat brain extracts to the incubation medium reduced tracer binding in proportion to its cyclo (His-Pro) content in a manner parallel to the cyclo (His-Pro) standards (Fig. 2). Furthermore, the properties of the cyclo (His-Pro)-like immunoreactivity was compared with authentic cyclo (His-Pro) by gel filtration and ionexchange chromatography. A trace

amount of (^3H -Pro)-cyclo (His-Pro) was added to the brain extracts to serve as a marker of the elution position of cyclo (His-Pro). Figure 3 shows the chromatographic profile obtained when a neutralized perchloric acid extract of rat brain was passed through DEAE (diethylaminoethyl)-cellulose (Panel A), CM (carboxymethyl)-cellulose (Panel B), and Sephadex G-25 columns. Endogenous cyclo (His-Pro) immunoreactivity (lower panel) co-eluted with exogenous (^3H -Pro)-cyclo (His-Pro) radioactivity (upper panel) in all three chromatographic procedures. The high pressure liquid chromatographic elution pattern of brain cyclo (His-Pro)-like immunoreactivity was also similar to authentic ^3H -cyclo (His-Pro) (retention time = 8 min).

B. Distribution of Cyclo (His-Pro) and its Precursor, TRH, in Rat and Monkey Brain

After developing a cyclo (His-Pro) RIA and establishing the co-identity of rat brain cyclo (His-Pro)-like immunoreactivity with that of authentic cyclo (His-Pro), we examined the regional distribution of this peptide in rat and monkey brains.

Cyclo (His-Pro) and TRH concentrations were determined first in seven regions (hypothalamus, hippocampus, cortex, striatum, mid-brain, cerebellum, and pons-medulla) of rat brain (Table 2). The data suggest an ubiquitous distribution of cyclo (His-Pro) in rat brain. The highest activity of cyclo (His-Pro) was found in hypothalamus (832.6 ± 58.6 fmols/mg protein) which was significantly ($p < 0.01$) higher than in cortex (601.6 ± 33.2 fmols/mg protein), the next highest region. The lowest concentrations of cyclo (His-Pro) were found in pons-medulla (397.9 ± 27.6 fmols/mg protein), which were statistically similar to cerebellar, mid-brain, and striatal cyclo (His-Pro) concentrations ($p > 0.2$), but lower than those in cortex or hippocampus ($p < 0.01$). The distribution pattern of TRH was quite distinct from that of cyclo (His-Pro). The hypothalamic concentration of TRH (2208.5 ± 155.3 fmols/mg protein) was the highest compared to any other region of rat brain (Table 2). At $p < 0.05$, the order of distribution of TRH in different regions was: hypothalamus > mid-brain > pons-medulla > striatum > cortex = hippocampus = cerebellum.

The analysis of cyclo (His-Pro) concentrations of 48 neuroanatomic loci of Rhesus monkey brain also revealed an ubiquitous distribution (Table 3). Monkey hypothalamus, like that of the rat, was richly endowed with this peptide (82 ± 8 fmols/mg protein). Highest concentrations of cyclo (His-Pro), however, were noted in the inferior olivary nucleus (157.7 ± 17.7), nucleus interpositus (103.4 ± 15.7), posterior raphe (99.5 ± 6.4), septal nucleus (95.4 ± 3.9) and pyriformis cortex (87.1 ± 15.8). Lowest cyclo (His-Pro) values (20.7 ± 56.5) were found in cerebral cortex (frontal, parietal and occipital) and thalamic nuclei. The measurement of TRH in the identical 48 neuroanatomic areas of the monkey brain revealed its highest concentration to be in hypothalamus ($151 - 161$ fmols/mg protein). The other areas found to be rich in TRH were: septal nucleus (78.2 ± 9.4), body of the caudate (59.2 ± 1.9), and nucleus accumbens (45.2 ± 10.2). There were a number of similarities in the distribution pattern of TRH in rat and monkey brains. In both of these species hypothalamus was rich in TRH whereas cerebellum and cortex contained very little TRH.

III. Regional Distribution of Pyroglutamyl Peptidase in Rat Brain

The first step in the formation of cyclo (His-Pro) from TRH is the cleavage of the pyroglutamyl moiety from TRH by the enzyme pyroglutamyl peptidase (Fig. 4). Therefore, in order to evaluate the possible relationship between cyclo (His-Pro) concentration and pyroglutamyl peptidase, the activity of this enzyme was determined in seven regions of the rat brain (Table 4). Enzymatic activity was distributed ubiquitously throughout the rat brain. Although cerebellar enzyme activity (650 ± 81 fmols/min/mg protein) was the highest, it was not statistically ($p > 0.05$) different from the activity in hypothalamus (641 ± 50), pons-medulla (530 ± 71) and mid-brain (500 ± 44). Furthermore, the ratio of the lowest activity (cortex 431 ± 48) to the highest activity (cerebellum) was only 0.72 ± 0.12 .

IV. Modulation of Endogenous Cyclo (His-Pro) Concentration in Rat Brain

Having established the presence of cyclo (His-Pro)-like immunoreactivity in rat and monkey brain, we explored the potential states in which the endogenous

concentration of this peptide might be modified. If cyclo (His-Pro) were a physiologically relevant peptide, its concentration, distribution or metabolism in brain should change in response to one or more of the different cyclo (His-Pro)-related physiological or pharmacological manipulations listed in Table 5. With this premise in mind, we have explored the possible change in endogenous cyclo (His-Pro) concentration in rat brain following prolonged alcohol consumption and altered thyroid status.

A. Chronic Alcohol Consumption Increases Cyclo (His-Pro)-Like Immuno-reactivity in the Rat Brain

Since cyclo (His-Pro) has been shown by us to attenuate ethanol induced sleep and also alcohol-mediated decreases in cerebellar cGMP content, we chose to study the effect of prolonged alcohol consumption on the endogenous level of brain cyclo (His-Pro). The concentrations of cyclo (His-Pro) and its precursor TRH in hypothalamic and extrahypothalamic regions of the rat brain, from control and alcohol treated rats, are depicted in Table 6. Chronic alcohol treatment for six weeks caused a 261% elevation in cyclo (His-Pro) in the hypothalamus, from 830 to 2170 fmols/mg protein ($p < 0.01$). Although the increment was smaller in magnitude (24%), cyclo (His-Pro) concentration was raised significantly also in the extra-hypothalamic brain ($p < 0.02$). In contrast, no significant changes in TRH concentration was observed in the hypothalamus. However, extrahypothalamic brain TRH concentration increased significantly from 60 ± 5 to 96 ± 8 fmols/mg protein ($P < 0.01$). The data herein indicate for the first time that ethanol administration to the rat produces an elevation in the brain concentration of cyclo (His-Pro), a peptide capable of reversing ethanol-induced sleep.

B. Hypothalamic Cyclo (His-Pro)-Like Immunoreactivity is Increased in Primary Hypothyroidism

Altered thyroid status has been shown to affect not only TRH metabolism but also prolactin secretion. Since cyclo (His-Pro) has been shown to inhibit prolactin secretion in vitro by us and others, we studied the effect of altered

thyroid status on brain cyclo (His-Pro) concentration in rats. The data presented in Table 7 shows that rats made hypothyroid by surgical thyroidectomy exhibited increased levels of cyclo (His-Pro)-like immunoreactivity in the hypothalamus (Sham-operated euthyroid control: 230.2 ± 16.6 pg/mg protein, hypothyroid: 324.1 ± 11.4). However, rats receiving exogenous thyroxine ($25 \text{ g T}_4/100 \text{ g body weight/day}$ for 2 weeks) did not show any change in the cyclo (His-Pro) concentration. In contrast, the hypothalamic concentration of TRH was not altered by changes in thyroid status, underscoring the specificity of the alteration of cyclo (His-Pro) concentrations.

V. Interaction Between Hypothalamic TRH-Receptor and Cyclo (His-Pro)

Because cyclo (His-Pro) and TRH affect the thermoregulatory process in the same general area and because the POA/AHA contains both TRH and TRH receptors, a possible mechanism for the hypothermic effect of cyclo (His-Pro) is the antagonism of TRH interactions with hypothalamic TRH receptors. The experimental data presented here, however, do not favor this hypothesis. The specific binding of (^3H -Pro)-TRH to hypothalamic synaptosomal membrane preparations ($58 \text{ fmols/mg protein}$) was decreased in a dose-dependent manner after the addition of increasing amounts of non-radioactive TRH. The concentration of TRH required for the half-maximal binding was $8 \times 10^{-10} \text{ M}$. In contrast, increasing quantities of cyclo (His-Pro) did not inhibit (^3H -Pro)-TRH binding. We conclude that cyclo (His-Pro) does not elicit the hypothermic response by blocking the receptor for TRH.

Publications Resulting From This Project

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TABLE 1

SPECIFICITY OF RABBIT ANTI-CYCLO (HIS-PRO) ANTIBODY

Peptide/Amino Acid	Immunoreactivity* (%)
Cyclo (His-Pro)	100.000
Cyclo (Pro-Pro)	0.781
Cyclo (Pro-Gly)	<0.005
Cyclo (Ala-Gly)	<0.005
His-Pro	6.892†
His	<0.005
Pro	<0.005
pGlu-His-ProNH ₂ (TRH)	0.119
pGlu-His-Pro (acid TRH)	0.036
pGlu-His	0.005
pGlu-His-Trp-Ser-Tyr-Gly-Leu	<0.005
Arg-Pro-GlyNH ₂ (LHRH)	

*Immunoreactivity based on dose needed to displace 50% of bound counts under conditions described in text. 100%=5.1 ng/ml.

†His-Pro is a relatively unstable peptide and has a tendency to cyclize to cyclo (His-Pro) non-enzymatically.

TABLE 2

REGIONAL DISTRIBUTION OF CYCLO (HIS-PRO) AND TRH IN RAT BRAIN (20)

Region	fmols Peptide/mg Protein*	
	Cyclo (His-Pro)	TRH
Hypothalamus	832.6 ± 58.6	2208.5 ± 155.3
Cortex	601.6 ± 33.2	78.1 ± 9.2
Hippocampus	571.7 ± 51.4	50.5 ± 10.4
Striatum	500.5 ± 44.8	126.0 ± 7.6
Mid Brain	478.3 ± 23.4	351.2 ± 36.7
Cerebellum	414.9 ± 26.0	31.5 ± 4.0
Pons-Medulla	397.9 ± 27.6	269.1 ± 15.1

*Mean ± SEM (n=21).

TABLE 3
REGIONAL DISTRIBUTION OF TRH AND CYCLO (HIS-PRO) IN MONKEY BRAIN

Region	Peptide (fmols/mg Tissue)*	
	Cyclo (His-Pro)	TRH
I. Brain Stem		
1. Pons-medulla		
Pontine nucleus	34.5 ± 5.2	3.7 ± 2.4
Inferior olivary nucleus	157.7 ± 17.7	16.8 ± 7.8
Magnocellular reticular nucleus	55.8 ± 6.2	11.6 ± 2.4
Raphe anterior	80.7 ± 13.3	20.7 ± 8.2
Raphe posterior	99.5 ± 6.4	18.5 ± 6.6
2. Mid Brain		
Superior colliculus	56.8 ± 9.2	3.1 ± 1.3
Inferior colliculus	60.0 ± 12.1	15.8 ± 5.1
Periaqueductal gray	71.3 ± 9.7	24.5 ± 6.9
Substantia nigra	49.9 ± 7.3	24.0 ± 7.0
II. Cerebellum		
Anterior cerebellum	38.6 ± 5.5	2.3 ± 0.6
Cerebellar hemisphere	42.4 ± 6.7	0.6 ± 0.2
Vermis (Lobule 8-10)	63.4 ± 7.2	0.3 ± 0.1
Dentate nucleus	53.7 ± 9.3	0.1 ± 0.1
Nucleus interpositus	103.4 ± 15.7	0.6 ± 0.1
Fastigial nucleus	66.7 ± 8.0	0.4 ± 0.2
III. Diencephalon		
1. Thalamus		
Lateral geniculate nucleus	35.5 ± 7.0	7.0 ± 1.1
Ventral posterior nucleus	50.6 ± 6.9	11.6 ± 3.1
Dorsomedial nucleus	50.5 ± 10.3	32.9 ± 7.2
2. Hypothalamus		
Anterior hypothalamus	81.4 ± 8.1	150.8 ± 19.2
Posterior hypothalamus	82.8 ± 7.3	161.3 ± 18.1
IV. Corpus Striatum		
Head of the caudate	27.3 ± 3.7	17.1 ± 1.3
Caudate body	65.8 ± 6.3	59.2 ± 19.3
Putamen, caudal	56.3 ± 7.6	18.7 ± 6.5
Putamen, rostral	48.8 ± 8.1	14.2 ± 2.4
Globus pallidus medial	54.8 ± 10.5	21.5 ± 6.1
Globus pallidus lateral	52.7 ± 6.9	16.5 ± 2.7
Nucleus accumbens	58.0 ± 11.7	45.2 ± 10.2
V. Limbic System		
Hippocampus	35.0 ± 6.4	13.1 ± 6.2
Amygdala	59.9 ± 4.7	15.5 ± 4.8
Septal nucleus	95.4 ± 3.9	78.2 ± 9.4
Cingulate gyrus anterior	42.4 ± 8.1	7.9 ± 1.1
Cingulate gyrus posterior	37.0 ± 7.3	2.8 ± 0.4
VI. Cortex		
Frontal tip	26.8 ± 8.2	1.4 ± 0.6
Superior frontal gyrus	32.7 ± 4.7	2.7 ± 0.9
Middle frontal gyrus	31.9 ± 4.0	3.7 ± 1.1
Inferior frontal gyrus	34.7 ± 2.4	1.9 ± 0.3
Temporal tip	53.5 ± 4.4	2.3 ± 0.5
Superior temporal gyrus	26.1 ± 3.6	1.4 ± 0.5
Middle temporal gyrus	34.7 ± 5.2	1.5 ± 0.4
Inferior temporal gyrus	48.4 ± 6.0	2.9 ± 1.3
Insular cortex	69.7 ± 8.1	6.0 ± 1.0
Pyriformis cortex	87.1 ± 15.8	11.8 ± 3.3
Motor cortex	20.7 ± 4.3	1.0 ± 0.1
Sensory cortex	24.2 ± 3.9	1.3 ± 0.6
Striate cortex	30.7 ± 5.6	1.6 ± 1.1
Peristriate cortex	26.0 ± 4.5	1.2 ± 0.6
Preoccipital cortex	56.5 ± 5.7	1.3 ± 0.4
VII. Spinal cord	56.8 ± 3.8	15.3 ± 3.1

*Mean ± SEM (n=5).

TABLE 4
REGIONAL DISTRIBUTION OF PYROGLUTAMYL PEPTIDASE
ACTIVITY IN RAT BRAIN

Region	Pyroglutamyl Peptidase Activity* (fmols/min/mg Protein)
Hypothalamus	641 ± 50
Cortex	431 ± 48
Hippocampus	449 ± 39
Striatum	432 ± 53
Mid-Brain	500 ± 44
Cerebellum	650 ± 81
Pons-Medulla	530 ± 71

*Mean ± SEM (n=7).

TABLE 5
BIOLOGICAL ACTIVITIES ASCRIBED TO
THYROTROPIN-RELEASING HORMONE
AND CYCLO (HIS-PRO)

- I. TRH-Related Activities of Cyclo (His-Pro)
 1. TRH-like activities
 - a. Antagonism of ethanol narcosis
 - b. Elevation of brain cGMP levels
 - c. Inhibition of food intake
 - d. Inhibition of cholesterol synthesis
 - e. Inhibition of abstinence syndrome in opiate dependent mice
 - f. Attenuation of ketamine-induced anesthesia
 2. TRH-opposite activities
 - a. Hypothermia in rats
 - b. Inhibition of *in vitro* prolactin secretion
- II. TRH-Unrelated Activity of Cyclo (His-Pro)
 - a. Inhibition of dopamine uptake
- III. Cyclo (His-Pro)-Unrelated Activities of TRH
 - a. Stimulation of thyrotropin secretion
 - b. Interaction with TRH-receptor
 - c. Behavioral effects, including piloerection, body tremor, and tail lifting
 - d. Inhibition of pentobarbital-induced sleep

TABLE 6
EFFECT OF ALCOHOL TREATMENT ON THE LEVELS OF
THYROTROPIN-RELEASING HORMONE AND CYCLO (HIS-PRO)
IN HYPOTHALAMIC AND EXTRAHYPOTHALAMIC AREAS
OF RAT BRAIN [19]

Treatment	Brain Area	fmols Peptide/mg Protein	
		TRH	Cyclo (His-Pro)
Control	Hypothalamic	1583 ± 214	830 ± 130
	Extrahypothalamic	60 ± 5	181 ± 12
Alcohol	Hypothalamic	1644 ± 250*	2170 ± 210†
	Extrahypothalamic	96 ± 8†	224 ± 14‡

All values are mean ± SEM (n=10).
 *p>0.05, †p<0.01, ‡p<0.02 compared to control.

TABLE 7
HYPOTHALAMIC CYCLO (HIS-PRO) IS ELEVATED IN HYPOTHYROID RATS

Thyroid Status	TSH (ng/ml)	Peptide (pg/mg Protein)	
		TRH	Cyclo (His-Pro)
Euthyroid (5)	377.2 ± 51.4	1342 ± 112	230 ± 17
Hyperthyroid (6)	undetectable	1512 ± 139	249 ± 14
Hypothyroid, 1* (6)	2880.1 ± 291.7*	1524 ± 71	324 ± 11*

*p<0.01 compared to euthyroid.

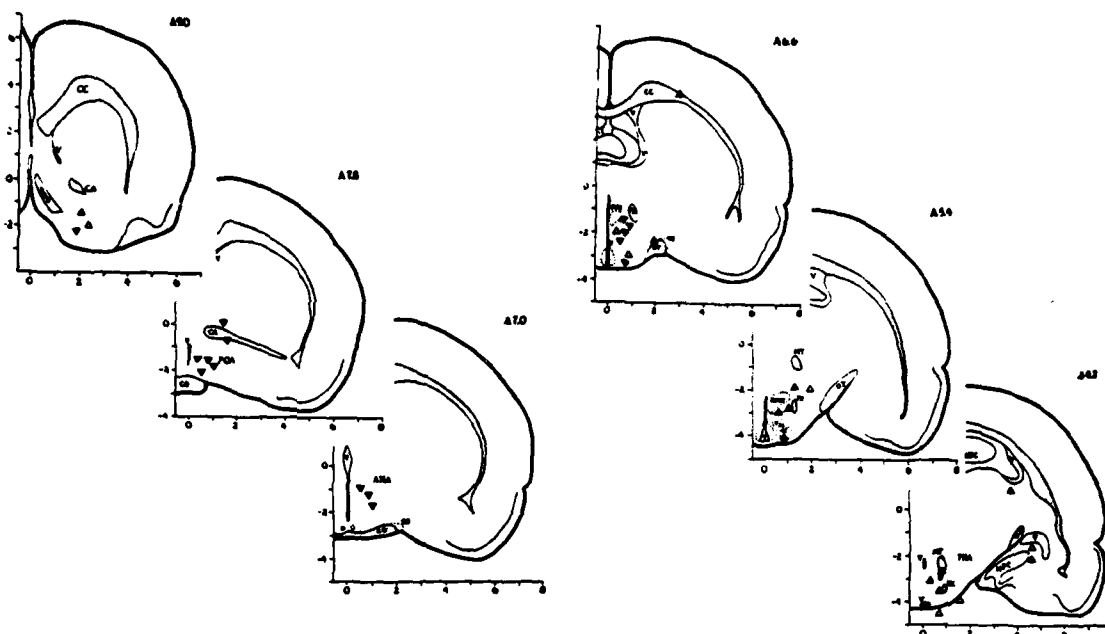


FIG. 1. Serial sections of the rat brain showing localization of sites where cyclo (His-Pro) was infused into the brain. Dark arrows (▼) indicate sites where there was a hypothermic response and open arrows (△) indicate sites where there was no effect.

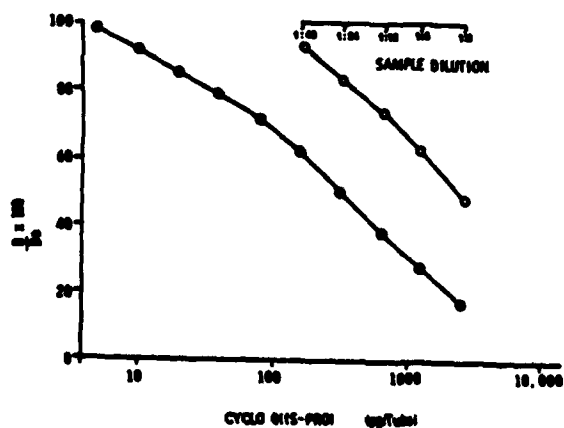


FIG. 2. Dose-response curve. Inhibition of antibody binding of $[^{125}\text{I}]$ -cyclo (His-Pro) by synthetic cyclo (His-Pro) and rat brain extract.

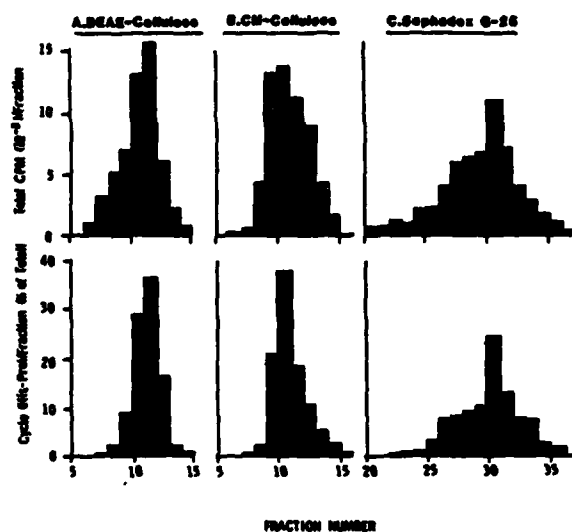


FIG. 3. DEAE-cellulose, CM-cellulose and Sephadex G-25 gel chromatography of [3 H]-cycle (His-Pro) and cycle (His-Pro)-like immunoreactivity in rat brain.

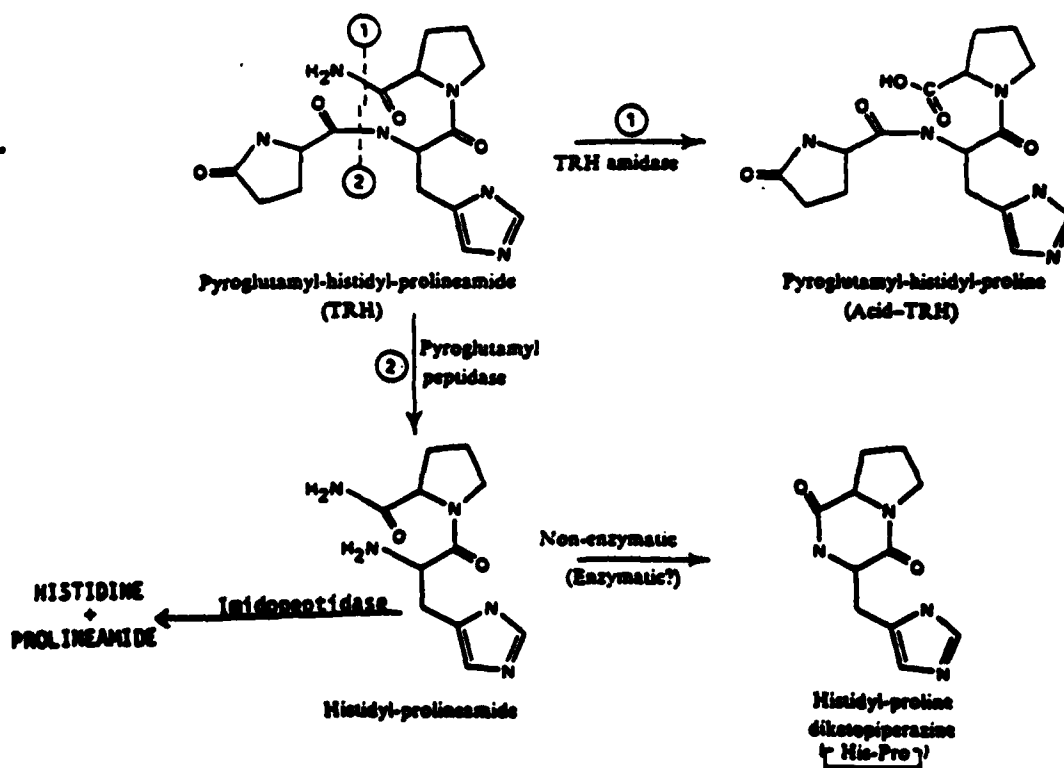


FIG. 4. Pathway of TRH metabolism in rat brain.

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